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Molecular Epidemiology in Cancer Risk Assessment and Prevention: Recent Progress and Avenues for Future Research

by Gerald N. Wogan

Molecular epidemiology is increasingly being applied in studies of cancer risks derived from exposure to environmental carcinogens of both endogenous and exogenous origins. Analytical methods have been developed that are capable of detecting and quantifying levels of covalent adducts of several important classes of carcinogens with cellular DNA and blood proteins. Methods of sufficient sensitivity and specificity to detect ambient levels of exposure are in current use. These are being used in studies related to tobacco use (polycyclic aromatic hydrocarbons, aromatic amines, tobacco-specific nitrosamines); dietary exposures (aflatoxins, *N*-nitrosamines, heterocyclic amines); medicinal exposures (cisplatin, alkylating agents, 8-methoxypsoralen, ultraviolet photoproducts); occupational exposures (aromatic amines, polycyclic aromatic hydrocarbons, oxides of ethylene and styrene, and vinyl chloride); and oxidative damage (8-hydroxyguanine, thymine glycol). Methodologic improvements together with their expanded use in feasibility studies continue to produce results that support the validity of this approach for detecting and quantifying exposure to carcinogens. Genetic markers are also being used to detect early biological responses in efforts to link carcinogen exposure to initiating events in the carcinogenesis process. These include, in addition to traditional cytogenetic markers (e.g., chromosomal aberrations, sister chromatid exchange, micronuclei), other alterations in chromosomal structure such as restriction fragment length polymorphisms, loss of heterozygosity, and translocation markers. Specific genetic changes have recently been identified as critical molecular events in the initiation and development of many cancers. Important among these are activation of oncogenes, especially those of the *ras* family, and inactivation of tumor-suppressor genes (e.g., *p53* and *Rb*) by point mutations and/or chromosomal deletions and other structural changes. Although some of these changes are known to occur in chemically induced tumors of experimental animals, the possible role of chemical carcinogens in the induction of genetic abnormalities in human cancers has yet to be determined. Continuing investigations employing the methods of molecular epidemiology promise to provide further evidence concerning these relationships. Future investigations employing newly developed molecular biological methods, in particular those based on polymerase chain reaction amplification of DNA, to identify alterations in DNA and chromosomal structure, combined with methods for characterizing exposure to carcinogens and early effects, have great potential for further elucidating the role of genotoxic agents in the etiology of human cancers and also for the development of strategies for their prevention.

Introduction

Molecular epidemiology of cancer involves the use of biomarkers of exposure and response in studies of exogenous or endogenous agents and/or host factors that play a role in human cancer etiology. The approach has the potential for serving multiple purposes, including improved accuracy of exposure measurement, identification of susceptible individuals in the presence of adverse exposures, detection of subclinical disease, more homogeneous classifications of disease, increased knowledge of disease pathogenesis, and improvements in methodology for preventive and therapeutic trials (*1*). As laboratory methodology for detection of exposure and response markers has advanced, the applications of molecular epidemiology have concomitantly increased in number and level of sophistication. Progress over the past 5 years, which is the time frame encompassed by information discussed here, has been substantial. The field has been the subject of many symposia, workshops, and other meetings, and various aspects have also been extensively reviewed (e.g., *1–8*). In addition, many facets of the field are discussed more fully in other papers in this volume.

At the previous meeting of this series, held in Helsinki in 1987, the major focus of discussion was methods for detection and characterization of genotoxic agents and their application in detection and prevention of human cancers. At that time, analytical procedures were available for detection and quantification of adducts formed between DNA or blood proteins and genotoxic carcinogens of a variety of structural types. In the intervening period, many of these have been modified to make them more sensitive, accurate, and easy. Methods for additional

[1]Division of Toxicology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

classes of carcinogen–DNA and –protein adducts have also been developed, and the list of carcinogen adducts for which analytical methods are available is now quite substantial. Methods of sufficient sensitivity and specificity to detect ambient levels of exposure are in current use. These are being used in studies of tobacco use (polycyclic aromatic hydrocarbons [PAH], aromatic amines, tobacco-specific nitrosamines); dietary exposures (aflatoxins, *N*-nitrosamines, heterocyclic amines); medicinal exposures (cisplatin, alkylating agents, 8-methoxypsoralen, ultraviolet photoproducts); occupational exposures (aromatic amines, PAH, oxides of ethylene and styrene, and vinyl chloride); and oxidative damage (8-hydroxyguanine, thymine glycol). Methodologic improvements, together with their expanded use in feasibility studies, continue to produce results that support the validity of this approach for detecting and quantifying exposure to carcinogens, and investigations designed to test their ability to measure exposures associated with elevated cancer risks are being conducted in increasing numbers.

Over the same interval of time, information concerning genetic alterations in cancers has increased significantly. Of particular relevance to molecular epidemiology are the genetic markers that are also being employed for detection of early biological responses, in efforts to link exposure to carcinogens to initiating events in the carcinogenesis process. These include, in addition to traditional cytogenetic markers (e.g., chromosomal aberrations, sister chromatid exchange, micronuclei), other alterations in chromosomal structure such as restriction fragment length polymorphisms, loss of heterozygosity, and translocation markers. Specific genetic changes have recently been identified as critical molecular events in the initiation and development of many cancers. Important among these are activation of oncogenes, especially those of the *ras* family, and inactivation of tumor-suppressor genes (e.g., *p53* and *Rb*) by point mutations and/or chromosomal deletions and other structural changes. Although some of these changes are known to occur in chemically induced tumors of experimental animals, the possible role of chemical carcinogens in the induction of genetic abnormalities in human cancers has yet to be determined. Continuing investigations employing the methods of molecular epidemiology promise to provide further evidence concerning these relationships.

Increased attention has been given to the importance of host susceptibility factors as determinants of individual cancer risk, in addition to markers of exposure and biological response, and substantial research effort has been devoted to developing markers of susceptibility. These include polymorphisms of carcinogen metabolism, of both genetic and environmental origin, which involve both activation and inactivation pathways. Markers for polymorphisms in activation pathways entail measurement of activity of members of the cytochrome P450 family of monooxygenases and of conjugating enzymes including glutathione-*S*-transferases, acetyltransferases, sulfotransferases, glucuronyltransferases and peroxidases. Markers of genetic polymorphisms in drug metabolism have also been developed and are being evaluated with respect to their relationships to cancer risks. These include, for example, the ability to metabolize debrisoquine (the *CYP2D6* locus) and transcriptional activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (the *CYP1A1* locus). The importance of inherited predisposition has also become increasingly evident as detailed genetic analyses have been done on specific genetically linked cancers such as retinoblastoma, Wilms' tumor, familial adenomatous polyposis, and the Li–Fraumeni syndrome. The importance of DNA repair deficiency, e.g., in xeroderma pigmentosum, as an inherited factor that predisposes individuals to elevated cancer risk has long been known.

The conceptual basis for the way in which molecular epidemiology can indicate the mechanisms on the basis of which these classes of markers have been developed, and the manner in which they are related to the carcinogenesis process, are summarized in Figure 1. This diagram illustrates the mechanistic paradigm of chemically induced carcinogenesis, on the basis of current knowlege of the process, in which exposure to chemical carcinogens leads to their absorption, metabolic activation, and subsequent covalent binding to cellular DNA and proteins. Measurement of DNA and protein adducts is designed to serve as a marker of exposure and biologically effective dose. The pattern of DNA adducts thus formed can be substantially altered by repair processes of varying efficiency and fidelity with respect to different carcinogens, tissues, and cell types. Replication of carcinogen–modified DNA is thought to result in the fixation of mutations that serve as initiating events in transformation. These can be detected through the use of traditional cytogenetic markers such as sister chromatid exchange or micronuclei in peripheral white blood cells (WBC), or through the detection of mutations at specific genetic loci, including *hprt*, HLA, or glycophorin in WBC and red blood cells. Mutations in the *ras* and *p53* genes can also be detected in the cells of end-stage tumors, where they are thought to be of functional significance in tumorigenesis. Increased cell proliferation, with clonal expansion of cells containing mutations that impart growth advantages is accompanied by the accumulation of further genetic alterations, such as additional point mutations, gene amplification, translocations, allelic loss, and chromosomal deletions. The number of genetic changes involved is not known, as suggested by the dashed arrows in the diagram.

Much of the information about molecular biomarkers that has accumulated in the recent past relates to the earliest and latest stages of the process, i.e., biomarkers of exposure and biologically effective dose, and biomarkers of altered structure and function or of clinical disease. In the latter category, mutations that cause activation of *ras* oncogenes and inactivation of the *p53* gene have been detected in tumors of diverse types and at varying frequencies. Certain relevant aspects of this information are summarized in subsequent sections. The nature of the mutations has supported the conjecture that they could have been induced by exposure to chemical carcinogens, but direct evidence of that relationship does not yet exist. The discovery of mutated *ras* and *p53* genes in a high proportion of adenomatous polyps representing early stages of colon cancer suggests that analytical methods of

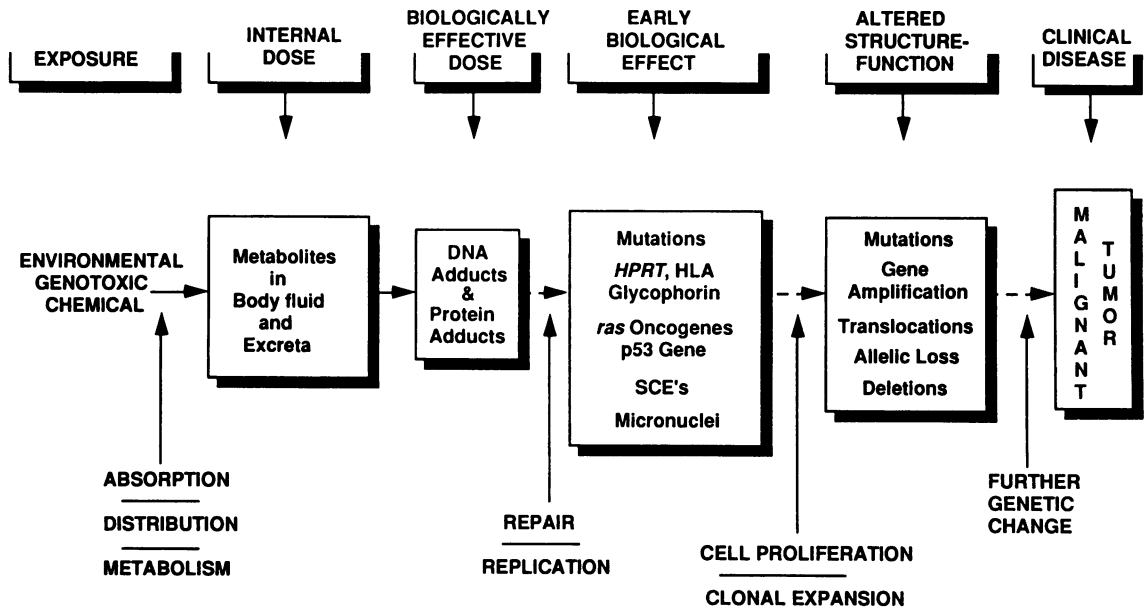

FIGURE 1. Conceptual basis for the development of biomarkers for use in molecular epidemiology. SCE, sister chromatid exchange.

adequate sensitivity may make it possible to detect such mutations in the early stages of other forms of cancer, including those associated with chemical exposures, and thus serve as markers of early biological effect. Little direct information is currently available, however, to support the conjecture that mutations observed in oncogenes or tumor-suppressor genes in end-stage tumors or in preneoplastic cells in humans are induced by carcinogens. Several investigators have sought to address this issue through the study of mutations induced at specific loci in the DNA of accessible surrogate cells in cancer chemotherapy patients exposed to large doses of genotoxic drugs or ionizing radiation. The results of such studies, which are summarized below, provide interesting and important perspectives on the use of the techniques of molecular epidemiology to detect and quantify genotoxic exposures.

DNA and Protein Adducts in Detection of Genotoxic Exposures

Analytical methods are now available for the detection and quantification of covalent adducts formed between DNA and proteins and genotoxic chemicals. Methods for DNA adduct analysis include immunoassays, ^{32}P-postlabeling, and physicochemical procedures, the latter being based on such properties as fluorescence or involving mass spectrometry (MS) and electrochemical analysis. Protein adducts in hemoglobin (Hb) and serum albumin can be analyzed by physicochemical methods, principally gas chromatography (GC)–MS, or by immunoassay. Collectively, methods are now available for the detection of DNA and/or protein adducts of many of the major classes of chemical carcinogens. These include PAH, particularly benzo[*a*]pyrene diol epoxide (BPDE), aromatic amines, nitrosamines of several types, aflatoxins, heterocyclic amines, medicinal agents of several classes, industrial chemicals (styrene and ethylene oxide), and products of oxidative DNA damage. These analytical procedures have been applied in studies of human populations exposed to genotoxic chemicals under a variety of circumstances. Space does not permit comprehensive review of this subject, but the following summary of recent publications will serve to indicate the directions these investigations have taken and the nature of findings obtained to date. For convenience, this information is grouped according to sources and types of exposure, including tobacco use, dietary, medicinal, and occupational exposures, and oxidative damage.

Adducts in White Blood Cell DNA and Blood Proteins

Many investigators have directed their efforts to detection of DNA and protein adducts in smokers or other users of tobacco. In a substantial fraction of these studies peripheral WBC were used as a source of DNA for analysis. Analysis by ^{32}P-postlabeling revealed the presence of multiple adducts in the WBC DNA of smokers, with total concentrations of one adduct in 10^8–10^{10} normal nucleotides (*9*). In another investigation using the same analytical procedure, the mean number of WBC DNA adducts per 10^8 nucleotides in smokers (31 ± 5.7) was significantly larger than that in nonsmokers (13 ± 1.6) (*10*). In a study designed to compare PAH–DNA adduct levels (measured

by enzyme-linked immunosorbent assay [ELISA]) with the frequency of sister chromatid exchange in peripheral WBC of lung cancer cases and control subjects, DNA adduct levels were not significantly related to active or passive cigarette smoking, nor to age, sex, ethnicity, or other parameters (*11*).

In a study of the association of Hb adducts of 15 amines with smoking status and type of tobacco smoked, levels of the 4-aminobiphenyl adduct varied significantly between nonsmokers and smokers of blond and black tobacco (*12*). Subsequently, it was found that levels of 4-aminobiphenyl–Hb adducts were related not only to tobacco type, but also to acetylation phenotype, with adduct levels being elevated in slow acetylators (*13*). Levels of the 4-aminobiphenyl–Hb adduct were also found to decline in the blood of smokers enrolled in a withdrawal program, decreasing from a mean of 120 ± 7 pg/g Hb to 34 ± 5 pg/g after 2 months (*14*). Hb adducts of 4-aminobiphenyl have also been detected in fetuses exposed to tobacco smoke *in utero*, indicating that the carcinogen or its active metabolite, *N*-hydroxy-4-aminobiphenyl, crosses the human placenta, and binds to fetal Hb in concentrations that are higher in smokers than nonsmokers (*15*). MS has been used in the analysis of tobacco-specific nitrosamine–Hb adducts in smokers, nonsmokers, and snuff dippers. Mean adduct levels (fmole 4-hydroxy-1-(3-pyridyl)-1-butanone/g Hb) were 517 ± 538 in snuff dippers, 79.6 ± 189 in smokers, and 29.3 ± 25.9 in nonsmokers (*16*). These results are the first reported measurements of tobacco-specific nitrosamine–Hb adducts in humans and indicate the potential usefulness of the analytical procedure for further characterization of cancer risks associated with cigarette smoking.

Analysis of DNA and protein adducts has also been successfully employed in the detection and quantification of exposure through dietary contamination by several carcinogens, including aflatoxins and PAH. Methods of several types have been employed for the detection of aflatoxin–serum albumin adducts. Levels of the aflatoxin B_1–albumin adduct detected by competitive radioimmunoassay were found to correlate well with aflatoxin ingestion, as well as with urinary excretion of the metabolite aflatoxin M_1 (*17*). A similar correlation between aflatoxin ingestion and albumin adduct level was obtained through application of an analytical method combining immunoaffinity chromatography and high-performance liquid chromatography (HPLC) for detection and quantification of the aflatoxin B_1–lysine adduct isolated from serum albumin (*18*). A similar method has been devised for analysis of the aflatoxin G_1–lysine adduct (*19*), which will be useful in further studies of exposure to aflatoxins in human populations. An immunoassay (ELISA) method has also been used in a survey of aflatoxin to exposure in different regions of the world, which showed that 12–100% of children and adults in various African countries have aflatoxin–serum albumin adducts, with levels of up to 350 pg aflatoxin B_1–lysine equivalent/mg albumin (*20*). Aflatoxin adducts were also detected by immunoassay and HPLC in DNA from human tissues after an acute poisoning incident in Southeast Asia (*21*).

An investigation of the effect of ingestion of large amounts of charcoal-broiled beef on formation of PAH adducts in peripheral WBC, detected by ELISA, showed a 3- to 6-fold elevation in the level of adducts in two of four subjects following a 1-week period of ingestion (*22*). These results indicate that dietary sources of PAH can contribute to the PAH–DNA adduct levels in WBC of some individuals. Methods for detecting DNA or protein adducts with other known dietary carcinogens are not yet available; however, two recent reports indicate progress in the development of such methods for the heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). A GC–MS method capable of detecting the parent amine liberated from Hb after hydrolysis was employed in the investigation of the covalent binding of ^{14}C-MeIQx to mouse Hb *in vivo* and *in vitro* (*23*). Although binding of the radioactive amine to Hb was demonstrable at relatively high doses, the analytical method was capable of detecting binding only at the highest doses used, and application of the GC–MS method to human Hb showed that if any MeIQx adducts were present the level was below the limit of detection of the assay (2 fmole MeIQx/mg Hb). Thus, the ability to detect exposure to this important class of dietary carcinogens awaits development of new analytical methods of greater sensitivity.

Studies of DNA and protein adducts in patients treated with drugs that damage DNA, in particular cancer chemotherapeutic agents, have been very informative, inasmuch as the dose levels are known and are higher than environmental exposures. Several such studies have been conducted on patients treated with cisplatin, for which immunoassays are available for the quantification of DNA adducts in WBC and tumor tissue DNA. Earlier studies showed that the extent of platinum–DNA adduct levels in WBC of ovarian cancer patients was directly related to disease response. Recently, a similar correlation between cisplatin–DNA adduct formation in peripheral WBC and disease response has been shown in patients with testicular cancer and a poor prognosis (*24*). Further, in a small cohort of refractory ovarian cancer patients treated with cisplatin or carboplatin, the adduct level in WBC DNA appeared to be more closely related to disease response than any previously identified prognostic variable (*25*). In another study of similar design (*26*), a nonlinear dependence of adduct levels on total dose of cisplatin was observed, which was attributed to removal of adducts. Within 21 hr of the first cisplatin infusion, 76% of adducts were removed, indicating the potential importance of this variable in the interpretation of adduct levels obtained in single blood samples collected in the course of biomonitoring studies. It is important to note, however, that adduct levels determined by ELISA do not agree well with those obtained by atomic absorption spectroscopy, and therefore the basis of the relationship between adduct levels and clinical response is still unclear.

Products of DNA alkylation have also been detected in WBC DNA of patients undergoing cancer chemotherapy. O^6-Methylguanine was detected in the WBC DNA of lymphoma patients receiving procarbazine, with levels

accumulating throughout the exposure period (*27*). Similarly, leukocyte DNA from blood of patients with Hodgkin's disease receiving a therapeutic regimen that included dacarbazine was found to contain O^6-methylguanine at levels ranging up to 0.45 fmole/μg DNA (*28*). The average extent of O^6-methylguanine formation 1 hr after treatment was 4.3×10^{-2} fmole/μg DNA per mg/kg dose. Recently, a ^{32}P-postlabeling method was developed to measure levels of 7-methylguanine in WBC DNA, and its application to the blood of healthy nonsmokers revealed the presence of 7-methylguanine at an average level of 2.5 residues/10^7 nucleotides (*29*). In cancer patients receiving dacarbazine or procarbazine at total doses of 1050–2800 mg, the observed mean adduct level was 57 residues/10^7 nucleotides. This method was suggested as that most suited to postlabeling of large and labile 7-alkylguanines in DNA.

DNA and protein adducts have been determined in patients treated with additional therapeutic regimens. An immunofluorescence assay involving a monoclonal antibody that recognizes 8-methoxypsoralen photoadducts was used to analyze DNA isolated from skin biopsies of psoriasis patients treated with 8-methoxypsoralen and ultraviolet A light. Adducts were detected in DNA of skin biopsies, but an ELISA using the same monoclonal antibody failed to detect adducts in the DNA of peripheral WBC (*30*). Hydroxyethylation of Hb was detected by GC–MS in the blood of patients treated with 1-(2-chloroethyl)-1-nitrosoureas, suggesting that measurement of (hydroxyethyl)valine in Hb may be a suitable monitor of exposure to hydroxyethylating agents during (chloroethyl)nitrosourea chemotherapy (*31*). Chromosomal alterations in WBC, urinary mutagenicity and excretion of PAH metabolites were studied in psoriatic patients undergoing coal-tar therapy (*32*): the results suggested that urinary mutagenicity levels as well as frequencies of sister chromatid exchange and chromosomal aberrations were related to the levels of exposure to coal tar.

Biological monitoring through the measurement of DNA and protein adducts has also been applied in relation to occupational exposures under a variety of conditions. In one such study, sister chromatid exchange and PAH–DNA adducts in peripheral blood cells were investigated in firefighters (*33*). These subjects had a significantly higher risk of detectable BPDE–DNA adducts than matched controls, with tobacco smoking and consumption of charcoal-broiled food and alcohol exerting confounding effects on both parameters studied. Coke-oven workers are occupationally exposed to relatively high levels of PAH and are at increased risk of lung cancer. Analysis of WBC DNA for PAH adducts revealed that 47% of workers had detectable levels of PAH–DNA adducts in their WBC, in comparison to 30% of controls (*34*). In both groups, smokers had significantly higher levels of adducts than did nonsmokers, but generally the correlation was not significant between PAH–DNA adducts in blood and the concentration of PAH in air or of 1-hydroxypyrene in urine. In another study of coke-oven workers and of residents of towns near cokeries, it was found that PAH–DNA adduct levels in WBC, determined by ^{32}P-postlabeling or ELISA, were similar in coke-oven workers and local residents but were two to three times lower in residents of rural areas (*35*). Furthermore, the results of this study showed that the levels of adducts in WBC DNA were not related linearly to ambient air levels of PAH, but other sources such as food may have been important contributors. Aluminum production plant workers are similarly exposed to high levels of PAH and may also be at increased risk of lung and bladder cancer. Analysis of WBC DNA for PAH adducts by ^{32}P-postlabeling revealed adduct levels of up to 7.1 adducts per 10^8 nucleotides in exposed individuals, compared to levels of up to 2.42 adducts per 10^8 nucleotides in controls (*36*). Higher DNA adduct levels were associated with elevated levels of exposure in two plants of different design.

Hb adducts have also been used to monitor occupational exposures in two recent studies. HPLC combined with synchronous scanning fluorescence spectroscopy provided a highly specific method for detecting covalently bound benzo[*a*]pyrene residues in both Hb and DNA in blood of coke–oven workers and in tissues from lung cancer patients (*37*). A GC–MS method for detecting Hb adducts with arylamines derived from urea and carbamate pesticides has recently been devised and validated in experimental animals; this may provide a means of monitoring occupational exposure to potentially hazardous components of pesticides (*38*).

The postulated importance of endogenously produced oxidative DNA damage in cancer has prompted efforts to develop methods that measure this damage. Earlier methods were based on measurement of thymine glycol and thymidine glycol in urine by HPLC, or of 8-hydroxy-2′-deoxyguanosine by HPLC with electrochemical detection. Immunoaffinity isolation of 8-hydroxy-2′-deoxyguanosine and -guanine from urine and quantification of the deoxyguanosine in DNA by polyclonal antibodies have recently been reported (*39*). The immunoaffinity procedure facilitated quantification of the urinary product, and the same antibody preparation was used to quantify 8-hydroxy-2′-deoxyguanosine levels in hydrolysates of cellular DNA with values comparable to those obtained by the earlier HPLC–electrochemical detection method. Quantification of levels of an oxidation product of thymine, 5-hydroxymethyluracil, in WBC DNA has been used to evaluate the relationship of dietary fat level and oxidative DNA damage in women at high risk for breast cancer (*40*). Levels of 5-hydroxymethyluracil were linearly related to dietary fat levels, and were 3-fold lower in a low-fat diet group than in controls. These results suggest that oxidative damage to DNA may be a useful marker of dietary fat intake.

Adducts in Target Tissue and Surrogate Cellular DNA

Interpretation of adduct levels in WBC DNA in relation to their implications for cancer risk entails the assumption that these are valid surrogates for DNA damage in cells of target tissues. In order to circumvent this assumption, increasing numbers of studies have focused on analysis of adduct levels in cellular DNA of putative target tissues. A

pilot study was conducted to determine whether a spectrum of adducts of varying chemical structures could be detected in small amounts of human DNA (*41*). Peripheral lung tissue was obtained at autopsy from trauma victims with known occupational and smoking histories, and different carcinogen–DNA adducts were detected by use of variety of sensitive analytical procedures. The results indicate that human lung contains a broad spectrum of carcinogen–DNA adducts and that molecular dosimetry studies should encompass both aryl and alkyl chemical carcinogens. Several studies have sought to correlate DNA adduct levels in human lung with cigarette smoking. Measurement of total adduct levels by ^{32}P-postlabeling in DNA of lung tissue revealed that smokers had higher levels of adducts than nonsmokers, that there was a linear relationship between adduct levels and cigarette consumption, and also that adduct levels declined in subjects who stopped smoking for 5 years to levels similar to those of nonsmokers (*42*). Subsequent studies of similar design, but with confirmational quantification of PAH–DNA adduct levels by ELISA, gave similar results and also showed a positive, linear correlation between DNA adduct levels and arylhydrocarbon hydroxylase activity in microsomes from the same tissues (*43,44*). These findings were extended in another investigation in which the tissue distribution of putative DNA adducts was investigated by ^{32}P-postlabeling; it was found that DNA adducts derived from smoking were present in kidney, bladder, esophagus, aorta, and liver, but levels were highest in lung and heart (*45*). A GC–MS method similar to that described earlier for analysis of Hb adducts has been devised to detect tobacco-specific nitrosamine adducts of DNA (*46*). Analysis of DNA from peripheral lung and tracheobronchial tissue collected at autopsy revealed higher levels of 4-hydroxy-1-(3-pyridyl)-1-butanone in both tissues from smokers than in those from nonsmokers and indicated the usefulness of this method for providing increasingly accurate information concerning DNA damage resulting from cigarette smoking. A further study was designed to investigate the feasibility of measuring DNA–carcinogen adducts in the lungs of nonsurgical patients (*47*). Endobronchial biopsies were obtained from patients undergoing routine bronchoscopy, and DNA isolated from them was analyzed for adducts by HPLC or ^{32}P-postlabeling, using a procedure selective for aromatic adducts. Adduct levels were found to be strongly associated with cigarette smoking history and with alcohol intake, but not with lung cancer independent of smoking history. The results further indicated the feasibility of this approach for biomonitoring genetic damage in bronchial epithelium. Tissue obtained through diagnostic bronchoscopy has also been used to evaluate the detection of micronuclei as an indicator of chromosomal damage resulting from cigarette smoking (*48*). The findings obtained suggested that micronuclei are a readily quantified, early–intermediate end point for detecting tobacco-initiated tracheobronchial carcinogenesis. Analysis of adduct levels in exfoliated oral mucosa cells has also been explored as a means of monitoring exposure of the oral cavity to genotoxic agents, with results leading to the conclusion that further improvements in adduct identification will be needed for ^{32}P-postlabeling to be a useful tool for monitoring exposure by this route (*49,50*).

Analysis of DNA from biopsy samples of urinary bladder by ^{32}P-postlabeling was used to determine the prevalence of carcinogen–DNA adducts in relation to cigarette smoking (*51*). Total mean adduct levels and the mean levels of several specific adducts were significantly higher in current smokers than in nonsmokers or ex-smokers. One of the specific adducts present at high levels was identified as *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl, confirming the association between cigarette smoking and DNA damage and suggesting a molecular basis for the initiation of bladder cancer by cigarette smoke. DNA hyperploidy in exfoliated urinary bladder cells was evaluated as a marker for biological response to exposure of the bladder in workers at high risk of exposure to various carcinogenic aromatic amines, primarily 2-naphthylamine (*52*). The prevalence of DNA hyperploidy was significantly elevated in a dose-related manner with increasing duration of exposure, and it was concluded that this parameter can serve as a marker for identification of workers at increased risk in occupational groups exposed to bladder carcinogens.

Recently, BPDE–DNA adducts were analyzed by synchronous scanning fluorescence spectrophotometry in pulmonary alveolar macrophages obtained by bronchoalveolar lavage of smokers, ex-smokers, and nonsmokers, with the objective of evaluating these cells as surrogates for target cells at risk for lung cancer in smokers (*53*). The results were interpreted to indicate that detection of BPDE–DNA adducts in alveolar macrophages, compared to other cell populations, was a sensitive, specific biomonitoring tool for assessing the internal dose of inhaled benzo[*a*]pyrene in the proximity of target cells of the respiratory tract. Because interindividual variations in the activities of DNA repair enzymes may be risk factors in the pathogenesis of lung diseases, the activities of O^6-methylguanine–DNA methyltransferase and uracil DNA glycosylase were measured in bronchoalveolar cells and peripheral WBC from healthy smoking and nonsmoking men (*54*). Wide interindividual variation was found in both enzyme activities, for which cigarette smoking was not entirely responsible. ^{32}P-Postlabeling analysis of urinary mutagens from smokers of black tobacco has implicated the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine as a major DNA damaging agent (*55*). These findings were obtained by analysis of DNA adducted *in vitro* by mutagens extracted from the urine of smokers in the presence of a metabolic activating system; and they extend the previous findings, described earlier, that indicate the additional importance of 4-aminobiphenyl as a risk factor in smokers of black tobacco.

Placenta, a readily available organ that responds to environmental exposures, has also been studied in several investigations as a surrogate tissue for biomonitoring of DNA adduct formation. BPDE–DNA adducts have been detected in a large proportion of placentas analyzed by HPLC in conjunction with synchronous scanning fluorescence spectrophotometry (*56*), additional information about the nature and extent of PAH–DNA damage was

obtained by immunoaffinity chromatography and ^{32}P-postlabeling (*57,58*). Comparable results were obtained in another study in which placental DNA was analyzed by ^{32}P-postlabeling and immunoassay. A positive dose–response relationship was found among smokers between levels of the smoking-related adducts and biochemical estimates of doses of maternal exposure to cigarette smoke during pregnancy (*59*). Carcinogen adducts have also been analyzed by ELISA in DNA isolated from fetal tissue and placentas from spontaneous abortions (*60*). PAH-derived adducts arising from sources other than cigarette smoke were found in a high proportion of fetal liver and lung samples; however, placenta was found not to be a good surrogate for fetal tissues with regard to adduct formation.

Multiple Exposure Markers in Relation to Cancer Risk

Few of the above studies incorporated the simultaneous use of multiple markers of exposure and biological effect for investigation of defined populations known to be at different levels of risk for a specific form of cancer. Such investigations will be imperative for advancement of the validation of molecular epidemiology as a predictive tool. Recent results of a series of investigations in smokers are therefore of particular importance and interest. In smokers of blond and black tobacco, previously shown to be at different levels of risk for bladder cancer (*61,62*), DNA adducts were measured in exfoliated urinary bladder epithelial cells by ^{32}P-postlabeling, Hb was analyzed for 4-aminobiphenyl adducts, and urine mutagenicity was determined (*63*). At least 4 of 12 DNA adducts detected appeared to be related to cigarette smoking, at levels two to nine times higher in smokers than in nonsmokers. Two DNA adducts were qualitatively similar to those observed in an earlier study of bladder biopsies, and one of these corresponded to *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl. The levels of the two DNA adducts were correlated significantly with the levels of 4-aminobiphenyl adducts in Hb and with the type and number of cigarettes smoked. In addition, levels of the putative 4-aminobiphenyl–DNA adduct were correlated with the mutagenic activity of the individual's urine. These combined data indicate the value of biomarkers applied in a noninvasive biomonitoring procedure in the molecular epidemiology of a specific form of cancer.

Markers of Oncogene Activation and Tumor-Suppressor Gene Inactivation

Development of malignancy is generally believed to involve multiple steps, in which cells acquire a series of genetic changes leading to progressive disruption of control mechanisms governing cell growth. Intensive research efforts continue to be directed toward elucidation of the series of events through which normal cells acquire a malignant phenotype. Accumulating evidence suggests that cancers of different origins may share certain common mechanisms involving activation of positively regulating factors (oncogenes) and loss or inactivation of suppressor genes, even though the precise molecular mechanisms leading to development of specific tumor types may differ. Cellular proto-oncogenes can be activated through several mechanisms, including mutations or other chromosomal structural changes, such as translocations, overexpression of normal or mutated proto-oncogenes, and amplification of proto-oncogene sequences. The development of rapid assay procedures for detecting *ras* mutations has led to the accumulation of a larger amount of information about the presence of these mutations in tumors than exists for any other genetic change. Much evidence has accumulated concerning the importance of *ras* genes in human cancers (*64*) and of *ras* oncogene activation in tumors induced experimentally in animals. Analysis of many activated *ras* genes isolated from tumor DNA or obtained through experimental manipulation of normal genes has revealed that they can be activated in two ways: through point mutations or by enhanced expression. All mutations identified to date with these methods are located in the codons for amino acids 12, 13, or 61 of the three *ras* genes. *ras* Gene mutations have been found in a variety of human tumor types, although the incidence varies greatly. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%); in thyroid tumors (50%); and in myeloid leukemia (30%). In contrast, they seem rarely to appear in tumors of neuroectodermal origin, differentiated lymphoid malignancies, or adenocarcinomas of the breast. An interesting and important aspect of *ras* gene mutations in human tumors is that they might have been induced by carcinogenic agents. This implies that the type of mutations found may provide information about the type of mutagen involved in the induction of the mutation, and therefore in the induction of the tumor. This is particularly relevant in cases when specific mutagens are already suspected of playing a role in the etiology of a particular cancer type, or when clear differences in mutational spectra are observed, as for example, in patients from different geographic regions. It is important in this context that cells in an end-stage tumor containing a *ras* mutation are selected, and therefore the type of mutation in them is also selected. This selection can be cell-type specific, and tissue-specific susceptibility for a particular mutagen as well as specificity of DNA damage and repair may influence the mutational spectrum observed in tumors. These factors underscore the importance of developing and applying analytical procedures of sufficient sensitivity and specificity to detect and quantify mutations in the earliest stages of tumorigenesis to make possible accurate assessment of the significance of environmental carcinogens as human cancer risk factors.

Activation of *ras* proto-oncogenes by point mutations has also been demonstrated in many types of tumors induced experimentally in animals by chemical carcinogens. Tumors of several tissues induced experimentally in mice by various chemical carcinogens have been analyzed for activated oncogenes (*65*). These include papilloma and carcinoma of the skin, fibrosarcoma, mammary car-

cinoma, lung carcinoma, T lymphoma, and hepatocellular tumors (adenoma and carcinoma). Those in the rat include mammary carcinoma, fibrosarcoma, lung carcinoma, renal carcinoma, neuroblastoma, glioblastoma, schwannoma, and hepatocellular carcinoma. Several interesting correlations have been drawn on the basis of information currently available. Most systems have a remarkable specificity for a particular oncogene—usually one member of the *ras* family. Activation of Ha-*ras* occurred with the highest frequency, followed by Ki-*ras*. There is a striking correlation between the tissue type affected in the tumors and the oncogene activated. In all epithelial tumors in mice (skin, mammary gland, and liver), Ha-*ras* is activated; while in mesenchymal tumors (lymphomas, fibrosarcomas, and renal), either Ki- or N-*ras* is activated. This preferential selection may be related to more prevalent expression of specific members of the *ras* family. With respect to correlations between the carcinogen used and the mutation found in the activated oncogene, the number of model systems studied is still too small and their characteristics too varied to support definitive conclusions; however, of the six possible base substitution mutations (including mutations in both DNA strands), four have already been found in one or more experimental systems. The $B6C3F_1$ mouse hybrid strain has been used extensively for bioassays of chemicals for carcinogenic activity. Analysis of Ha-*ras* oncogenes in liver tumors induced in these animals by lifetime feeding of carcinogens of diverse structural types revealed that the patterns of activating mutations specific for each carcinogen were consistent with the interpretation that they arose from direct interaction of the activated carcinogen with the Ha-*ras* gene. Thus, an extensive data base supports the significance of *ras* genes activated by point mutations in the development of both human and experimentally induced tumors. It remains to be determined whether these mutations represent early initiating events in tumorigenesis or play a role at later stages of the process. In either case, many lines of available evidence indicate that *ras* mutations in themselves are not sufficient to produce fully developed malignant tumors. As noted above, development of malignancy is generally believed to involve multiple steps, in which cells acquire a series of genetic changes leading to progressive disruption of the control mechanisms governing cell growth.

In this context, information concerning the *p53* gene in the development of human tumors is also very pertinent in the context of molecular epidemiology (*66,67*); this is discussed in detail elsewhere in this volume. Although the cellular *p53* gene was originally thought to be an oncogene, more recent evidence indicates that the normal (i.e., unmutated) gene product actually functions as a tumor suppressor. A high prevalence (75%) of allelic deletion of the *p53* gene, coupled with mutational inactivation of the remaining allele, has been observed in colorectal cancers, and loss of *p53* function in a majority of human osteosarcomas has been correlated with gross structural rearrangements in the *p53* gene. Allelic deletion of the chromosome 17 region harboring the *p53* gene has also been reported in many other cancers, including those of the lung (60%), breast, ovary, cervix, adrenal cortex, bone, bladder, and brain. Thus, current evidence indicates that mutations in the evolutionarily conserved codons of the *p53* tumor-suppressor gene are common in diverse types of human cancer. The *p53* mutational spectrum differs in cancers of the colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues, and hematopoietic tissues. Analysis of these mutations can provide clues to the etiology of these diverse tumors. Transition mutations predominate in colon, brain, and lymphoid malignancies, whereas G–C to T–A transversions are the most frequent substitutions observed in the lung and liver. Mutations at A–T base pairs are seen more frequently in esophageal carcinomas than in other solid tumors. Most transitions in colorectal carcinomas, brain tumors, leukemias, and lymphomas are at CpG dinucleotide mutational hot spots. G to T transversions are dispersed among numerous codons. In liver tumors in people from geographic areas in which both aflatoxin B_1 and hepatitis B virus are cancer risk factors, most mutations are G–C to T–A transversions at the third nucleotide pair of codon 249. These differences may reflect the etiological contributions of both exogenous and endogenous factors to human carcinogenesis (*66*).

The identification of mutated *ras* and *p53* genes in end-stage tumors and the availability of sensitive analytical procedures for their detection provide avenues for the possible development of biomarkers for the presence of these genetic lesions in preclinical disease states. Lung tumors from smokers provide a unique opportunity for investigating oncogene activation in a human tumor believed to be initiated by chemical carcinogens in tobacco smoke. A recent investigation of activated proto-oncogenes in lung tumors from smokers revealed the presence of activated oncogenenes in 86% (10/12) of DNA samples from smokers, 8/10 of which contained mutated *ras* oncogenes, which appear to play a role in metastasis in pulmonary adenocarcinomas (*68*). In a second investigation of possible relationships between exposure to tobacco smoke and presence of *ras* mutations, more mutations (all in codon 12 of K-*ras* oncogenes) were found in adenocarcinomas from smokers (8/27) than in tumors from nonsmokers (2/27) (*69*). These results suggest that exposure to carcinogens in tobacco smoke may be an important factor in the induction of point mutations in the K-*ras* oncogene in these tumors. A high frequency (35%) of mutations in codons 12 and 61 of the H-*ras* oncogene has also been detected in human oral carcinomas related to tobacco chewing in India (*70*).

Mutations in the *p53* gene have recently been identified in 11 of 18 bladder tumors, and also in cells of the urinary sediment in each of three patients tested (*71*). The *p53* mutations were the first genetic alterations demonstrated to occur in a high proportion of primary invasive bladder cancers, and detection of this mutation in exfoliated cells indicates a possible biomonitoring strategy for early detection of the genetic damage.

Detection in serum of oncogene proteins has been used as a biomarker in the molecular epidemiology of occupational carcinogenesis (*72*). The *ras* oncogene-encoded p21 protein has been used as a prototype, since it is known to

be activated by occupational carcinogens, is frequently found in human tumors of occupational concern, and appears to be expressed early in the disease process, at least in certain instances, allowing the possibility of early detection and intervention.

Mutations Observed *in Vivo* in Humans

Methods have been developed for the detection of mutations at several selected loci in human WBC and erythrocytes, as discussed in detail elsewhere in this volume (*73*). Measurements of mutation frequency at the *hprt* locus in cultured T lymphocytes have been particularly informative in this regard, and a significant body of data has accumulated about the frequency of background mutations and induced mutations under a variety of exposure conditions. Studies of mutation frequency in patients undergoing chemotherapy or radiation have been particularly informative, and several can serve for illustrative purposes. *hprt* Mutations were measured in a prospective study of multiple sclerosis patients receiving intravenous infusions of cyclophosphamide, a known mutagen (*74*). Variant frequencies were measured in T cells cultured from blood samples collected at intervals after initial treatment. Two weeks after the first infusion of cyclophosphamide, the variant frequencies were significantly increased, but by 4 weeks they had returned to pretreatment levels. Subsequent treatments produced similar transient increases, but by 7–13 weeks after treatment the variant frequencies of all patients had returned to pretreatment levels. The transient nature of the response suggested selection *in vivo* against cyclophosphamide-induced thioguanine-resistant cells. A similar design was used to determine the time course of appearance and persistence of elevated frequencies of *hprt* variants in cancer patients treated with X–irradiation therapy (*75*). Twelve patients treated with 180–200 cGy/day, 5 days/week for 3–7 weeks were studied. During the third and fourth weeks of treatment, there was a significant (5- to 15-fold) elevation of variant frequency in all patients, and by 6–37 weeks after treatment variant frequencies had fallen to near pretreatment levels, showing a similar transience in the induced mutation frequency, as observed in the previous study. In the context of use of this parameter as a biomarker of genotoxic exposures, the transient nature of the response as well as its dose–response characteristics emphasize the importance of careful selection of sampling times following exposure.

Research is also in progress to determine whether the spectrum of mutations induced at the *hprt* locus in human T cells can be used to identify the nature of the mutagens responsible for their induction. Currently available data, which are being consolidated in a repository at the University of North Carolina, will be of particular value in this regard. The nature of these data, kindly provided by T. Skopek (personal communication) can be summarized as follows. In the combined *in-vitro–in-vivo* database, base substitution mutations have been identified at 193 sites (of a total of 657); a total of 257 mutations have been observed, of which 87% were missense, 11% nonsense, and 2% involved no amino acid change. At the eight CpG sites in the gene, six G–C to A–T transition mutations were located at position 151, and nine at positions 501–509. Base-substitution mutations have been observed *in vivo* in healthy subjects (58 sites), in Lesch–Nyhan patients (47 sites), and in people with low exposure (smokers with background mutant frequencies; 86 sites). Although limited overlap was observed in the sites of mutation between the three groups, the preponderance of mutations were G–C to A–T transitions or G–C to T–A transversions, the distribution of which suggests the presence of mutational "hot spots" in the gene. Collectively, the data suggest that the *hprt* locus has the potential to serve as a sensitive detector of base-substitution mutations in humans (T. Skopek, personal communication).

Studies of the effects of chemotherapeutic agents on the frequency of glycophorin A "null" variant erythrocytes *in vivo* are also of interest in this context (*76*). An assay based on the enumeration of variant erythrocytes lacking expression of an allelic form of glycophorin A was used to study blood samples from patients obtained prior to, during, and following chemotherapy for malignant disease. With certain therapeutic regimens, the variant frequencies gradually rose during therapy, reached a maximum (2- to 7-fold higher than normal) at or shortly after the end of therapy, then declined to pretherapy levels during early posttherapy, with a further decline to normal within six months. Previous investigations utilizing the same assay showed an increased frequency of variant erythrocytes *in vivo* in survivors of the atomic bombings in Hiroshima and Nagasaki, as well as in smokers.

The observation of *ras* mutations in WBC of patients following cytotoxic therapy for lymphoma is of particular relevance to molecular epidemiology (*77*). The occurrence of myelodysplastic syndrome and acute myeloblastic leukemia following cytotoxic therapy for cancer is well recognized, and *ras* mutations are commonly observed in patients with these diseases. To determine whether these mutations could be used as early markers of secondary disease, the incidence of *ras* mutations was determined in WBC of patients in complete remission from lymphoma who had been treated with standard regimens of chemotherapy or combined chemo-radiotherapy. DNA from peripheral WBC was analyzed for mutations in the three members of the *ras* gene family following polymerase chain reaction (PCR) amplification. Mutations were found in 9 of 70 patients who had received no treatment for 6 months to 14.5 years, and who showed no sign of residual disease. All patients were hematologically normal at the time of analysis, indicating that clones of mutant *ras*-bearing cells may be detected prior to any overt sign of disease. These findings indicate the power of PCR–DNA amplification for the detection of site-specific mutations in functionally important genes, and also offer promise for the development of additional, informative, early-response biomarkers based on similar analytical approaches.

Research Needs and Avenues for Future Development

The foregoing summary indicates that the molecular epidemiology of cancer is a research domain of considerable promise; however, the field is still at a relatively early stage of development, and the promise remains largely unfulfilled. While substantial progress has been made in certain areas, especially in the development of laboratory analytical procedures for biomarkers of several types, further improvements are still needed in order to facilitate their ready incorporation into the design of epidemiologic studies of cancer risks. With respect to analytical methods, improvements in accuracy, reliability, and interpretability (i.e., validation in experimental models) are required. Modifications of methods to reduce costs will be crucial to their use in large-scale population studies. Well-designed studies of populations of normal individuals will be required to characterize the distributions of markers in different age and sex groups. Similarly, intraindividual variability with respect to tissue localization and persistence of markers, as well as interindividual variability in genetic and acquired susceptibility factors must be characterized to make possible valid interpretations of data generated through molecular epidemiologic studies. Increasing applications of such biomarkers will also raise serious bioethical issues that will need to be addressed.

Some specific avenues of research that would facilitate progress can be identified. Existing analytical methods for DNA and protein adduct measurements, should be modified, when possible, to reduce their costs and thereby increase their applicability. In their current form, most methods are too complex, labor intensive, and costly to permit their use in population-based investigations. Development of new approaches of greater sensitivity and specificity can also be contemplated: for example, the use of accelerator MS for the detection of DNA adducts (*78*). Generic methods for detecting DNA adducts would also greatly facilitate progress in this area, inasmuch as current methods tend to be adduct specific or group specific, requiring analysis on an adduct-by-adduct basis. An example of such a method, based on application of the UvrABC DNA repair system, has been suggested (*79*). In the arena of markers of genetic damage, dramatic advances are being made in DNA diagnostics, combining molecular techniques and automation (*80*). Applications of the PCR amplification of DNA have led to the development of analytical procedures for detecting sequence changes in DNA with very high sensitivity and specificity. When coupled with radioisotopic, enzyme or fluorophore labeling of oligonucleotides, or with denaturing gradient gel electrophoresis, PCR-based analysis has been shown to be capable of detecting mutations at levels approaching background frequencies [see for example Keohavong et al. (*81*)]. Furthermore, one such procedure, PCR with oligonucleotide ligation, has recently been fully automated (*82*). Importantly, many of these methods can be applied for the analysis of archival pathologic tissue specimens, making possible retrospective studies based on such material. Many of these methods have been developed in connection with the human genome sequencing program, and further rapid progress can be anticipated. Incorporation of these advances into the development of new biomarkers of early response and disease, as they become available, will greatly facilitate progress of the field.

In addition to methodologic advances, opportunities for further advancement require increased collaborative research efforts, involving the active participation of specialists in epidemiology together with laboratory scientists in the design, execution, and interpretation of studies of cancer risks. Of perhaps greater importance for the longer term is the cross-disciplinary training of molecular epidemiologists, who must be educated at a professional level in the necessary aspects of basic science and also in epidemiology. Development of a cadre of investigators with this training will be necessary for molecular epidemiology to fulfill its promise to improve identification of cancer risks and implementation of preventive strategies.

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